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(54) Title: HYDANTOIN-RACEMASE

(57) Abstract: The instant invention is directed to a rec-hydantoin-racemase from *Arthrobacter aureus* DSM 3747. Furthermore, the gene encoding for the racemase and plasmids, vectors and microorganisms comprising this gene are to be protected. Use in a process for the production of amino carboxylic acids or derivatives thereof.

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Hydantoin-Racemase

Description

The instant invention is directed to a hydantoin-racemase from *Arthrobacter aurescens* (DSM 3747, hyuA).

5 The production of optically pure amino carboxylic acids is of growing interest in agrochemical, food and pharmaceutical industry. In particular, the enzymatic hydrolysis of hydantoins is an attractive method for the synthesis of D- and L-amino acids with regard to low-cost
10 starting material and complete turnover of substrate.

Several hydantoin degrading micro-organisms have been isolated and the enzymatic conversion of 5'-monosubstituted hydantoins was studied in detail (Syldatk and Pietzsch, "Hydrolysis and formation of hydantoins" (1995), VCH
15 Verlag, Weinheim, pp. 409-434; Ogawa et al., J. Mol. Catal. B: Enzym. 2 (1997), 163-176; Syldatk, C., May, O., Altenbuchner, J., Mattes, R. and Siemann, M. (1999) Microbiol. hydantoinases - industrial enzymes from the origin of life? Appl. Microbiol. Biotechnol. 51, 293-309).
20 The asymmetric bio-conversion to either L- or D-amino acids consists of 3 steps:

- (i) chemical and/or enzymatic racemization of 5' substituted hydantoins,
- (ii) ring opening hydrolysis achieved by a
25 hydantoinase and
- (iii) carbamoylase catalysed hydrolysis of the N-carbamoyl amino acid produced in the second step.

The chemical racemization of hydantoins proceeds via
30 enolisation. The velocity depends on the electronic nature of the residue at the 5'-position (Ware, Chem. Rev. (1950),

46, 403-470) but usually, the racemization is a very slow process. For example, at room temperature and pH 8.5 only about 10 % of L-IMH is racemized to D-IMH in 20 hour (Syldatk et al., "Biocatalytic production of amino acids and derivatives" (1992), Hanser publishers, New York, pp. 75-176). The rate of racemization is increased by a very basic pH (>10) and high temperature (>80 °C).

At physiological conditions a high rate of racemization is achieved by hydantoin-specific racemases. So far, hydantoin racemases have been purified and characterised from *Arthrobacter* (Syldatk et al., "Biocatalytic production of amino acids and derivatives" (1992), Hanser publishers, New York, pp. 75-176; Syldatk et al., "Hydrolysis and formation of hydantoins" (1995), VCH Verlag, Weinheim, pp. 409-434) and a *Pseudomonas* species (Watabe et al., *J. Bacteriol.* (1992a), 174, 3461-3466; Watabe et al., *J. Bacteriol.* (1992b), 174, 7989-7995). Only the latter is also characterised in terms of nucleotide sequence and genetic organisation.

It was, therefore, an object of this invention to provide another rec-hydantoin-racemase, which is able to racemize hydantoins under physiological conditions with an acceptable rate for their implementation in a process for the production of enantiomerically enriched amino carboxylic acids on industrial scale.

Providing the recombinantly derived hydantoin-racemase from *Arthrobacter aurescens* DSM 3747 (Seq. 4) is responsible for the dispense from above mentioned task. Especially, the racemase according to the invention can advantageously be incorporated in a large scale process for the production of enantiomerically enriched amino carboxylic acids. The feasibility of providing the racemase in a recombinant manner is the clue for acceptance of this process in view of economic efficiency.

Furthermore, a gene (Seq. 3) encoding for the racemase according to the invention is protected. The gene with relation to the framework of this invention is seen as a group of genes comprising all possible genes encoding for the protein in question according to the degeneration of the genetic code.

In another embodiment this invention encompasses plasmids, vectors and micro-organisms, which comprise the gene of instant invention. Within the framework of this invention all plasmids, vectors and micro-organisms which could advantageously be used to carry out the invention and are known to the skilled worker are incorporated herewith. Especially, those mentioned in Studier et al., Methods Enzymol. 1990, 185, 61-69 or those presented in brochures of Novagen, Promega, New England Biolabs, Clontech or Gibco BRL are deemed to be suitable. More applicable plasmids, vectors can be found in:

DNA cloning: a practical approach. Volume I-III, edited by D. M. Glover, IRL Press Ltd., Oxford, Washington DC, 1985, 1987;

Denhardt, D. T. and Colasanti, J.: A surey of vectors for regulating expression of cloned DNA in E. coli. In: Rodriguez, R.L. and Denhardt, D. T (eds), Vectors, Butterworth, Stoneham, MA, 1987, pp179-204;

Gene expression technology. In: Goeddel, D. V. (eds), Methods in Enzymology, Volume 185, Academic Press, Inc., San Diego, 1990;

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold SpringHarbor, N. Y.

In addition, primers useful for the amplification of the gene of the invention in a PCR are protected similarly. Primers which are feasible are for example:

S1137 5'-AGAACATATGAGAATCCTCGTGATCAA-3' (Seq. 1)

S1138 5'-AAAACTGCAGCTAGAGGTACTGCTTCTCTG-3' (Seq. 2)

Furthermore, all other primers which could serve to carry out this invention and which are known to the artisan are deemed to be useful in this sense. The finding of a suitable primer is done by comparison of known DNA-sequences or translation of amino acid sequences into the codon of the organism in question (e.g. for Streptomyces: Wright et al., Gene 1992, 113, 55-65). Similarities in amino acid sequences of proteins of so called superfamilies are useful in this regard, too (Firestone et al., Chemistry & Biology 1996, 3, 779-783). Additional information can be found in Oligonucleotide synthesis: a practical approach, edited by M.J. Gait, IRL Press Ltd, Oxford Washington DC, 1984; PCR Protocols: A guide to methods and applications, edited by M.A. Innis, D.H. Gelfound, J.J. Sninsky and T.J. White. Academic Press, Inc., San Diego, 1990. Those strategies are incorporated by reference herewith.

Another embodiment of this invention is the use of the racemase of the invention in a process for the production of amino carboxylic acids or derivatives thereof. Preferably, it is used according to the invention in a process for the production of enantiomerically enriched derivatives. Most preferably, the use is conducted in a covalent enzyme-membrane-reactor (DE19910691.6) or after non-covalent or covalent immobilisation to solid carriers (DE 197 033 14).

In order to prove the enzyme function, the gene was amplified by PCR from plasmid pAW16 using the primers S1137 and S1138 and placed under the control of a rhamnose promoter provided by the expression system pJOE2702. The resulting plasmid was designated pAW210 (Fig. 1). The E. coli cells harbouring pAW210 exhibited specific hydantoin racemase activities up to a maximum of 60 U/mg in crude

cell extracts (Fig. 2). The racemase activity was determined in crude extracts by polarimetry using 3 mM L-BH as substrate (Teves et al., Fresenius' J. Anal. Chem. 1999, 363, 738-743). An abundant protein of 31 kDa, representing approximately 10 % of the total cellular protein, was detected by SDS-PAGE analysis in rhamnose induced cells and was mainly in the soluble fraction of the crude cell extracts.

The plasmid pAW210 in *E. coli* JM109 was used for purification of the racemase. A two step procedure consisting of ammonium sulfate fractionation and MonoQ anion exchange chromatography was accomplished as described down under. The racemase was purified 10-fold to homogeneity, with 35 % overall recovery (Tab. 1).

Table 1: Purification of the racemase HyuA from *E. Coli* JM109 pAW210

Step	Volume [ml]	Protein- con. [mg/ml]	Volumetric activity [U/ml]	Specific activity [U/mg]	Total activity [U]	Purifica- tion [-fold]	Yield [%]
Crude extract	3	22.4	604	26.9	1812	1.0	100
(NH ₄) ₂ SO ₄	2.5	7	317	45.2	792	1.7	44
MonoQ ^{a)}	8.0	0.8	64	313.0	512	11.6	28

^{a)} Protein was purified on MonoQ in 4 separate runs using 4 mg for each run.

The specific activity of the purified enzyme was determined by standard enzyme assay with D-Benzylhydantoin as substrate at 313 U/mg. In potassium phosphate buffer, pH 7.0 with 25 % glycerol, the purified enzyme could be stored for at least 6 months at -20 °C without noticeable loss of activity.

The matrix assisted laser desorption ionisation spectrum (MALDI) of the purified racemase gave a peak at a molecular mass of 25078.7. This is in good agreement with the calculated value of 25085 Da in contrast to the SDS-PAGE electrophoresis which gave a relative molecular mass of 31 kDa for the racemase monomer. On a calibrated column of superose 12 HR, the relative molecular mass of the native enzyme was estimated to be approximately 170 kDa \pm 25. Due to the small subunit of 25 kDa and inaccuracy of the gel filtration method within this range the native enzyme is suggested to be either a hexamer, heptamer or octamer.

The effect of pH and temperature on the enzyme activity and stability are illustrated in Fig. 3-5. The pH optimum was determined between pH 8.0 and 9.0. Consequently, all standard assays were performed at pH 8.5. The optimum temperature for racemization of L-BH was around 55 °C, however the stability of the enzyme under assay conditions (Tris, pH 8.5) was only maintained up to 45 °C.

Racemization of the 5-substituted hydantoins BH, IMH and MTEH by HyuA was examined (Tab.2).

Table 2: Substrate specificity of HyuA

Substrate	Conc.	Relative Activity *)
	[mM]	[%]
L-MTEH	0.9	7
D-MTEH	0.9	8
L-BH	0.9	100
D-BH	0.9	95
L-IMH	0.9	13
D-IMH	0.9	12

*) 100 % racemase activity corresponds to 313 μ /mg determined by standard assay

L- and D-BH gave the highest rates of activity, whereas the L- and D-isomer of MTEH were rather poorly racemised

indicating that aromatic hydantoins were preferred as substrates.

The K_M values of IMH and BH could not be determined due to the limited solubility of the substrates. Instead the
5 initial velocities at different concentrations of L-MTEH were measured. The kinetic plot (Fig.6) showed that the racemase is inhibited by the substrate L-MTEH. Even at low substrate concentrations (> 5 mM) inhibition is observed.

The microorganism *Arthrobacter aurescens* used for the
10 invention was deposited at Deutsche Sammlung für Mikroorganismen under the accession number DSM 3747.

Examples:

- Bacterial strains, plasmids and growth conditions.** *E. coli* JM109 (Yanisch-Perron et al., Gene (1985), 33, 103-109) was used for cloning, sequencing and expression the *hyuA* gene from *Arthrobacter aurescens* DSM 3747 (Groß et al., Biotech. Tech. (1987), 2, 85-90). *E. coli* strains were cultivated in 2xYT liquid broth or on 2xYT agar (Sambrook et al., Molecular Cloning: A Laboratory Manual (1989), Cold Spring Harbour Laboratory Press, New York). The media were supplemented with 100 µg/ml ampicillin to select plasmid carrying strains. The cultures were grown at 37°C, for *hyuA* expression the growth temperature was reduced to 30°C.
- General protocols.** All of the recombinant DNA techniques used were standard methods (Sambrook et al., Molecular Cloning: A Laboratory Manual (1989), Cold Spring Harbour Laboratory Press, New York). PCR reactions were performed with Taq DNA polymerase by following the recommendation by Roche Molecular Biochemicals. DNA sequencing was done from pUC-subclones with automated laser fluorescens DNA sequencer (Pharmacia LKB, Freiburg) by using AutoRead™ sequencing kit and M13 forward and reverse primer.
- Expression of *hyuA* in *E. coli*.** The racemase gene was amplified by PCR using the primers S1137 (5'-AGAACATATGAGAATCCTCGTGATCAA-3') and S1138 (5'-AAAACTGCAGCTAGAGGTACTGCTTCTCTG-3') and pAW16 as template (Wilms et al., J. Biotechnol. (1999), 68, 101-113). The fragment was inserted between the NdeI and PstI sites of the expression vector pJOE2702 (Volff et al., Mol. Microbiol. (1996), 21, 1037-1047) to create plasmid pAW210. Expression was induced by addition of 0.2 % rhamnose to

cultures at an optical density of 0.3 at 600 nm. After 6 h, cells corresponding to OD₆₀₀ of 10 were harvested, washed and resuspended in 1 ml desintegration buffer (0.07 M potassium phosphate, pH 7.0) and lysed by sonification (Ultrasonics sonicator, microtip, 2 x 30 s, duty cycle 50 % pulsed). Clarified extracts were obtained by centrifugation at 14000 rpm for 10 min.

Enzyme assays. Racemization of L-BH was measured by ORD-polarimetry (Model 341, Perkin Elmer Bodenseewerk, Überlingen, Germany) at a wavelength of 295 nm in the standard assay for racemase enzyme activity. 3 mM L-BH was dissolved in 0.1 M Tris, pH 3 at 45 °C in an ultrasonic waterbath, cooled to room temperature and the pH adjusted to pH 8.5 with 3 M NaOH. To 1 ml substrate solution 0.1 ml enzyme, diluted in 0.1 M Tris, pH 8.5, was added and the change in optical rotation determined at 37 °C by polarimetry (Teves et al., Fresenius' J. Anal. Chem. 1999, 363, 738-743). The racemization of MTEH and IMH by HyuA was determined at substrate concentrations of 0.9 mM and recorded by ORD at 253 nm and 334 nm. The specific activities were calculated from initial reaction rates which were determined according to Teves et al. (1999). For determination of enzyme activity by HPLC, 1 mM L-IMH was dissolved as described above. The mixture containing 900 µl enzyme solution was incubated 5 min at 37°C. The reaction was stopped by addition of 400 µl 14 % trichloroacetic acid and centrifugation in an Eppendorf centrifuge at full speed. 100 µl of the sample were diluted with 0.9 ml 0.1 M TrisHCl, pH 8.5, and D-IMH and L-IMH in the supernatant were separated by HPLC (Thermoseparation Products, Darmstadt, Germany) by injection of 20 µl sample into a Chiralpak WH-column (0.46x25 cm; Daicel Chemicals Industries LTD, Griesheim, Germany). The column was equilibrated with 0.25 mM CuSO₄, pH 5.5. The flow rate was 1 ml/min at 50°C

and IMH detected at 254 nm. The chemical racemization of the substrate was taken into account. Racemization of 1 μ M substrate per minute was defined as one unit enzyme.

Purification of recombinant hyuA. For the preparation of
5 crude extract, cells from 300 ml culture of rhamnose
induced E. coli JM109 pAW210 were resuspended in 3 ml
desintegration buffer and disrupted 3 times by french press
(Amico, SLM Instruments Inc, Illinois, USA) at a pressure
of 600 bar. Solid $(\text{NH}_4)_2\text{SO}_4$ was gradually added to the cell-
10 free extract to a concentration of 1.5 M and stirred 2 h at
4 °C. The precipitate formed was removed by centrifugation
(Sorvall) and discarded. Another 0.7 M $(\text{NH}_4)_2\text{SO}_4$ was added
to the supernatant. The second precipitate obtained by
centrifugation was resuspended in buffer A (10 mM potassium
15 phosphate, pH 6.5) and applied to a MonoQ[®] HR 5/5 column
equilibrated in buffer A and eluted with a linear gradient
of 0 to 1.0 M NaCl in buffer A. HyuA was eluted at a
concentration of 0.37 M NaCl. Peak fractions were pooled
and dialyzed against desintegration buffer, glycerol was
20 added to a final concentration of 25 % and stored at
-20 °C.

Protein characterisation. Sodium dodecyl sulfate
polyacrylamide gel electrophoresis (SDS-PAGE) was done
25 according to the method of Laemmli (Laemmli, Nature (1970),
227, 680-685). Protein concentrations were determined by
the method of Bradford (Bradford, Anal. Biochem. (1976),
72, 248-254) using the Biorad protein assay dye reagent
concentrate. Standard curves were generated with bovine
30 serum albumin. The M_r of native protein was determined by
gel filtration using superose12HR column as described
previously (Wilms et al., J. Biotechnol. (1999), 68,
101-113), the column was equilibrated and eluted with
buffer consisting of 0.1 M potassium phosphate and 0.1 M
35 NaCl, pH 7. The pH profile of the purified racemase was

measured between the pH range 7.0 to 9.5 in Tris buffer. The substrate was dissolved in 0.1 M Tris, pH 3 at 45 °C using an ultrasonic waterbath. After cooling to room temperature, the pH was adjusted to the desired pH with sodium hydroxide and enzyme activity was determined using the standard assay. The reaction temperature optimum of purified racemase was determined using temperatures between 25 and 65 °C in the standard assay. The stability of the enzyme was measured after preincubation at temperatures between 25 and 70 °C for 15 minutes in the presence of desintegration buffer and 0.1 M Tris buffer, pH 8.5, respectively. The increased chemical racemization at high pH and temperatures, respectively, was considered. The effect of EDTA, DTT, HgCl₂ and iodoacetamid on HyuA was tested by incubation of respective substance (10 mM) and purified enzyme (12 µg) in desintegration buffer (final volume 20 µl) at 30°C. After 1 h specific activities were determined by the standard enzyme assay.

PCT

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0-3	Applicant's or agent's file reference	990097 AM
1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	7
1-2	line	9-11
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
1-3-2	Address of depositary institution	Mascheroder Weg 1b, D-38124 Braunschweig, Germany
1-3-3	Date of deposit	
1-3-4	Accession Number	DSMZ 3747
1-4	Additional Indications	NONE
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

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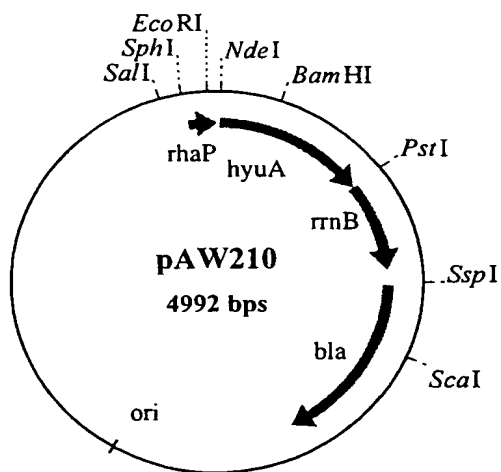
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Hydantoin-Racemase**Claims**

1. rec-Hydantoin-racemase from *Arthrobacter aurescens* DSM 3747.
- 5 2. Gene encoding for the racemase according to claim 1.
3. Vector comprising the gene according to claim 2.
4. Microorganism comprising the gene according to claim 2.
5. Primer for a gene according to claim 2.
- 10 6. Use of the racemase according to claim 1 in a process for the production of amino carboxylic acids or derivatives thereof.
7. Use according to claim 6 in a process for the production of enantiomerically enriched compounds.
- 15 8. Use according to claim 6 and/or 7, characterised in that, the process is conducted in a enzyme-membrane-reactor.

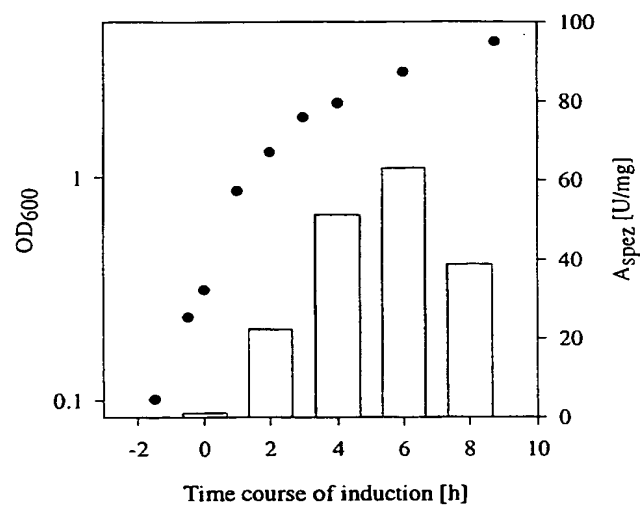
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Fig. 1



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Fig. 2



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Fig. 3

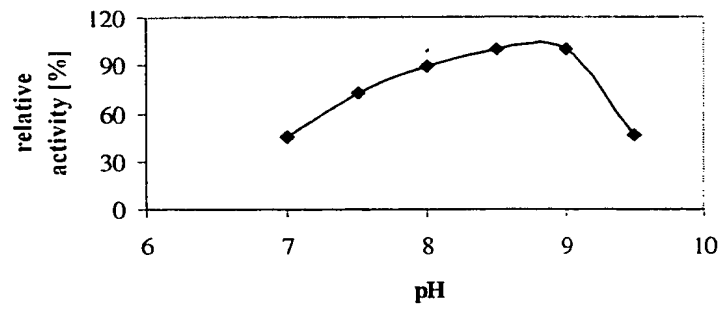
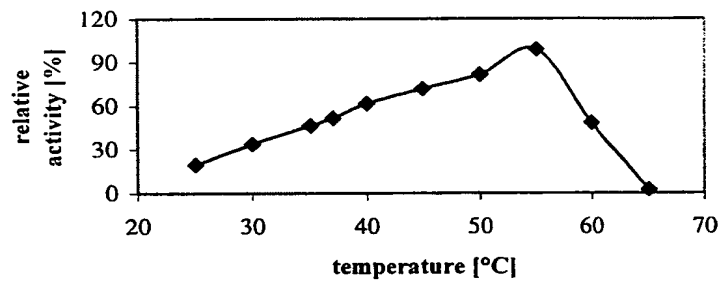
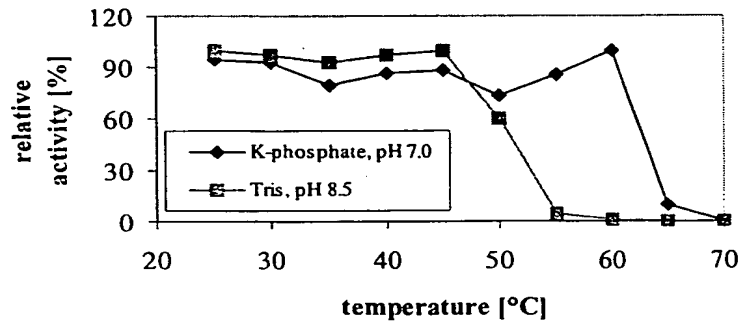


Fig. 4

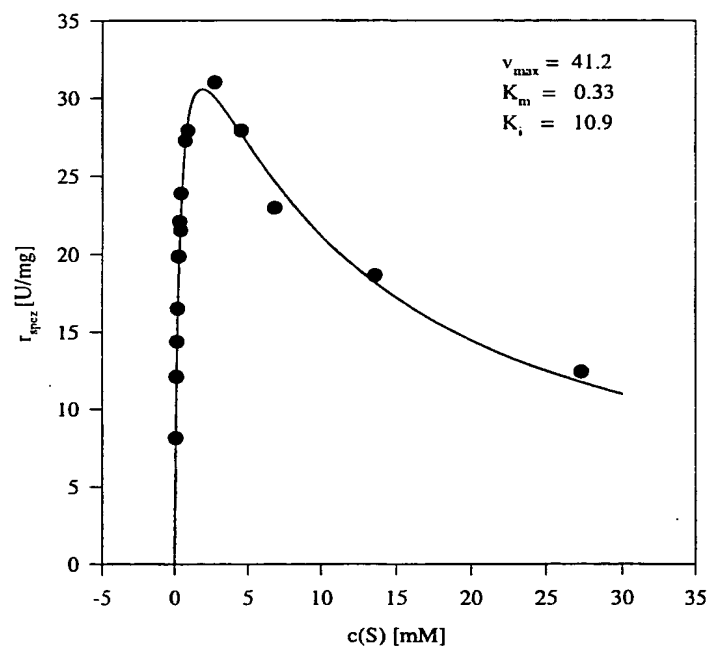


5 Fig. 5



4/4

Fig. 6



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3

	Ser	Val	Ala	Asp	Ala	Ala	Gln	Gln	Val	Val	Ala	Thr	Gly	Thr	Ile	Ile	
				20					25					30			
5	Ser	Ala	Ile	Asn	Pro	Ser	Arg	Gly	Pro	Ala	Val	Ile	Glu	Gly	Ser	Phe	
			35					40					45				
	Asp	Glu	Ala	Leu	Ala	Thr	Phe	His	Leu	Ile	Glu	Glu	Val	Glu	Arg	Ala	
		50					55					60					
10	Glu	Arg	Glu	Asn	Pro	Pro	Asp	Ala	Tyr	Val	Ile	Ala	Cys	Phe	Gly	Asp	
		65				70					75					80	
	Pro	Gly	Leu	Asp	Ala	Val	Lys	Glu	Leu	Thr	Asp	Arg	Pro	Val	Val	Gly	
					85					90					95		
15	Val	Ala	Glu	Ala	Ala	Ile	His	Met	Ser	Ser	Phe	Val	Ala	Ala	Thr	Phe	
				100					105					110			
	Ser	Ile	Val	Ser	Ile	Leu	Pro	Arg	Val	Arg	Lys	His	Leu	His	Glu	Leu	
20			115					120					125				
	Val	Arg	Gln	Ala	Gly	Ala	Thr	Asn	Arg	Leu	Ala	Ser	Ile	Lys	Leu	Pro	
		130					135					140					
25	Asn	Leu	Gly	Val	Met	Ala	Phe	His	Glu	Asp	Glu	His	Ala	Ala	Leu	Glu	
		145				150					155					160	
	Thr	Leu	Lys	Gln	Ala	Ala	Lys	Glu	Ala	Val	Gln	Glu	Asp	Gly	Ala	Glu	
				165						170					175		
30	Ser	Ile	Val	Leu	Gly	Cys	Ala	Gly	Met	Val	Gly	Phe	Ala	Arg	Gln	Leu	
				180					185					190			
	Ser	Asp	Glu	Leu	Gly	Val	Pro	Val	Ile	Asp	Pro	Val	Glu	Ala	Ala	Cys	
35			195					200					205				
	Arg	Val	Ala	Glu	Ser	Leu	Val	Ala	Leu	Gly	Tyr	Gln	Thr	Ser	Lys	Ala	
		210					215					220					
40	Asn	Ser	Tyr	Gln	Lys	Pro	Thr	Glu	Lys	Gln	Tyr	Leu					
		225				230					235						

INTERNATIONAL SEARCH REPORT

Int. l. Application No
PCT 00/08580

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/90 C12P41/00 C12N15/61 //(C12P41/00,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBL, STRAND, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PIETZSCH M ET AL.: "Isolation and characterization of a new, non pyridoxal-5'-phosphate dependent hydantoin racemase" DECHEMA BIOTECHNOLOGY CONFERENCES, vol. 4 pt. A, 1990, pages 259-262, XP000991534	1
Y	the whole document	2-8
Y	US 5 827 717 A (BOMMARIUS ANDREAS ET AL) 27 October 1998 (1998-10-27) column 1, line 65 -column 2, line 3 column 3, line 25-55 column 4, line 38-53 claims 1-6	2-7

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *G* document member of the same patent family

Date of the actual completion of the international search

16 March 2001

Date of mailing of the international search report

23/03/2001

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van de Kamp, M

INTERNATIONAL SEARCH REPORT

 Intl. Application No
 PCT 00/08580

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>BOMMARIUS A S ET AL.: "Membrane bioreactors for the production of enantiomerically pure.alpha.-aminoacids" , CHIRALITY IND. (1992), PP 371-97. EDITOR(S): COLLINS, ANDREW N.; SHELDRAKE, G. N.; CROSBY, J. PUBLISHER: WILEY, CHICHESTER, UK. XP000991099 paragraph '20.1! - paragraph '20.2! paragraph '20.4.1.3! paragraph '20.5!</p>	8
A	<p>WILMS B ET AL.: "Cloning, nucleotide sequence and expression of a new l-N-carbamoylase gene from Arthrobacter aurescens DSM 3747 in E. coli" JOURNAL OF BIOTECHNOLOGY, vol. 68, no. 2-3, 19 February 1999 (1999-02-19), pages 101-113, XP004164275 abstract page 102, left-hand column, line 1-18 page 102, left-hand column, line 41 -right-hand column, line 18 page 112, right-hand column, line 16-23</p>	1-8
A	<p>PIETZSCH M ET AL.: "Immobilization of hydantoin cleaving enzymes from Arthrobacter aurescens DSM 3747 - effect of the coupling method on the stability of the L-N-carbamoylase" PROG. BIOTECHNOL., STABILITY AND STABILIZATION OF BIOCATALYSTS, vol. 15, 1998, pages 517-522, XP000991023 abstract</p>	8
A	<p>WOHLFAHRT G ET AL: "Immobilization of hydantoin cleaving enzymes;" DEHEMA BIOTECHNOL. CONF., vol. 5 pt. A, 1992, pages 45-48, XP000991533 page 45, line 1-10 page 48, line 1-17</p>	8
P,X	<p>WIESE A ET AL.: "Hydantoin racemase from Arthrobacter aurescens DSM 3747: heterologous expression, purification and characterization" JOURNAL OF BIOTECHNOLOGY, vol. 80, no. 3, 14 July 2000 (2000-07-14), pages 217-230, XP004214557 the whole document</p>	1-8

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT 00/08580

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>OLIVER M ET AL.: "Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine"</p> <p>NATURE BIOTECHNOLOGY, vol. 18, no. 3, March 2000 (2000-03), pages 317-320, XP002154849 abstract page 320, left-hand column, line 9-13</p>	1-7
E	<p>WO 00 58449 A (DEGUSSA ; CALIFORNIA INST OF TECHN (US)) 5 October 2000 (2000-10-05) page 8, line 25 -page 9, line 17 example 2 claims 20,26,31</p>	1-8

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/00/08580

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5827717 A	27-10-1998	DE 4316928 A	24-11-1994
		AT 196776 T	15-10-2000
		DE 59409542 D	09-11-2000
		EP 0625571 A	23-11-1994
		ES 2150452 T	01-12-2000
		JP 6343462 A	20-12-1994
		SG 46729 A	20-02-1998
		US 5516660 A	14-05-1996
WO 0058449 A	05-10-2000	AU 4178800 A	16-10-2000